Current Biology, Vol. 13, 1571–1582, September 16, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.cub.2003.08.030

Eph/Ephrin Signaling Regulates the Mesenchymal-to-Epithelial Transition of the Paraxial Mesoderm during Somite Morphogenesis

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Summary

Background: During somitogenesis, segmental patterns of gene activity provide the instructions by which mesenchymal cells epithelialize and form somites. Various members of the Eph family of transmembrane receptor tyrosine kinases and their Ephrin ligands are expressed in a segmental pattern in the rostral presomitic mesoderm. This pattern establishes a receptor/ligand interface at each site of somite furrow formation. In the *fused somites (fss/tbx24)* mutant, lack of intersomitic boundaries and epithelial somites is accompanied by a lack of Eph receptor/Ephrin signaling interfaces. These observations suggest a role for Eph/Ephrin signaling in the regulation of somite epithelialization.
Results: We show that restoration of Eph/Ephrin signaling in the paraxial mesoderm of *fss* mutants rescues most aspects of somite morphogenesis. First, restoration of bidirectional or unidirectional EphA4/Ephrin signaling results in the formation and maintenance of morphologically distinct boundaries. Second, activation of EphA4 leads to the cell-autonomous acquisition of a columnar morphology and apical redistribution of β-catenin, aspects of epithelialization characteristic of cells at somite boundaries. Third, activation of EphA4 leads to nonautonomous acquisition of columnar morphology and polarized relocalization of the centrosome and nucleus in cells on the opposite side of the forming boundary. These nonautonomous aspects of epithelialization may involve interplay of EphA4 with other intercellular signaling molecules.
Conclusions: Our results demonstrate that Eph/Ephrin signaling is an important component of the molecular mechanisms driving somite morphogenesis. We propose a new role for Eph receptors and Ephrins as intercellular signaling molecules that establish cell polarity during mesenchymal-to-epithelial transition of the paraxial mesoderm.

Introduction

Somites are the most obvious manifestation of vertebrate embryonic metamerism. Somitogenesis involves the specification of groups of cells within the paraxial mesoderm as segments and the subsequent transformation of these segments into epithelial somites separated by intersomitic furrows. Major progress has been made in the identification of genetic factors that regulate the establishment of a segmental pattern prior to furrow formation (reviewed in [1, 2]). Few studies, however, have addressed the molecular mechanisms that drive the morphogenetic processes occurring during somite epithelialization.

Prior to somite formation, cells within each segment of the rostral presomitic mesoderm (PSM) acquire either anterior or posterior character. Anterior-posterior polarity within the presumptive somite is evident as segmental patterns of gene expression regulated by periodic activation of Notch signaling [3, 4]. Boundaries of gene expression between cells with anterior identity and cells with posterior identity are subsequently translated into morphological furrows between somitic cells that undergo the mesenchymal-to-epithelial transition. Genes segmentally expressed in the rostral PSM are therefore candidates for being involved in the morphogenetic processes leading to somite formation.

The Eph family of receptor tyrosine kinases and their Ephrin ligands show Notch signaling-dependent segmental expression in the rostral PSM [5, 6]. Eph proteins are membrane-spanning receptor proteins involved in intercellular signaling in many morphogenetic processes during embryonic development (reviewed in [7–9]). Ephrin ligands for these receptors are classified into two groups according to their association to the cell membrane. Class A Ephrins are tethered to the membrane by a GPI linkage and preferentially bind EphA receptors, whereas Class B Ephrins (which preferentially bind EphB receptors) are transmembrane ligands with an intracellular domain. EphA4 is the only receptor that binds both classes of ligands [10]. A notable feature of this pathway is that bidirectional signaling cascades are triggered upon Eph/Ephrin interaction (reviewed in [11]). Signaling downstream of Eph receptors is called forward signaling, whereas signaling downstream of Ephrins is called reverse signaling. Molecules that directly or indirectly interact with the intracellular domain of Eph receptors are in most cases regulators of the cytoskeleton and cell adhesion (reviewed in [12]) as well as other transmembrane receptors [13, 14]. One frequent consequence of Eph/Ephrin signaling is repulsion between cell populations [15], and roles in cell sorting and boundary formation in the zebrafish hindbrain have been demonstrated [16, 17]. Eph receptors and Ephrins are therefore excellent candidates for driving the morphogenetic events associated with somite epithelialization.

We have previously shown that disruption to Eph/Ephrin signaling results in a lack or aberrant formation of intersomitic furrows [18]. In this paper, we have used the mutant *fused somites* (*fss*) as an in vivo system to study the role of Eph/Ephrin signaling during somite morphogenesis. *fss* encodes Tbx24, a T box transcription factor involved in maturation of the PSM [19]. *fss* mutants lack anterior-posterior polarity within presumptive segments of the rostral PSM and fail to form somites

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[2]This paper is dedicated to the memory of Nigel Holder.

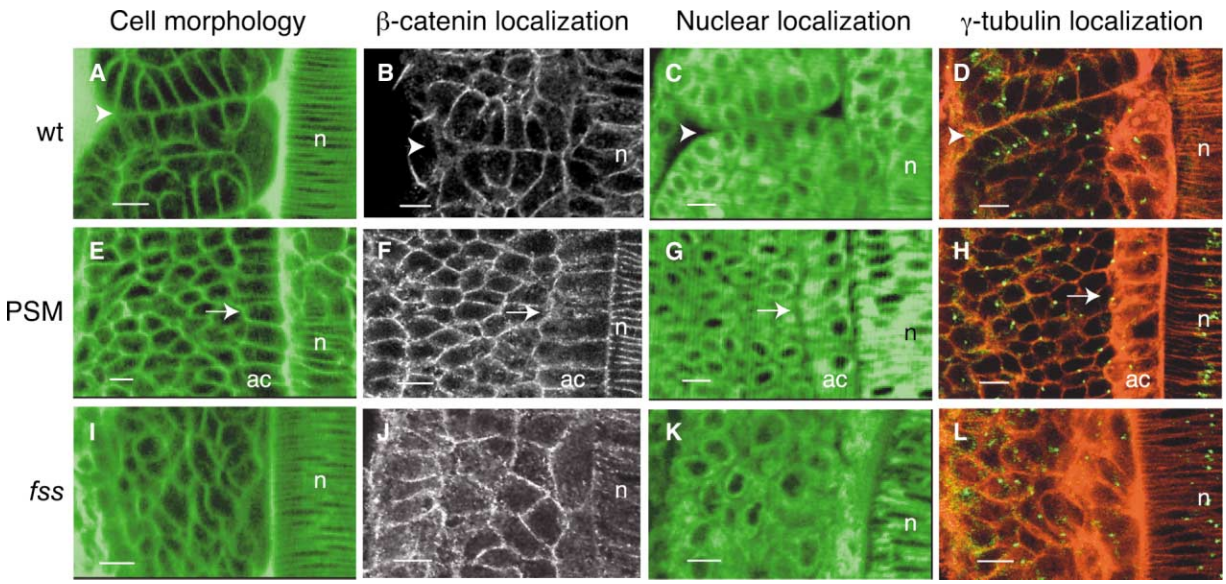

Figure 1. Cells Undergo Mesenchymal-to-Epithelial Transition at Somite Boundaries

(A–L) Dorsal views of the left-sided paraxial mesoderm of embryos labeled with Bodipy ceramide (which reveals cell morphology; [A], [E], and [I]) or with Bodipy 505-515 (which reveals nuclear position, [C], [G], and [K]) or immunostained for β-catenin ([B], [F], and [J]) or for γ-tubulin (which labels centrosomes) and stained with phalloidin (which labels actin) ([D], [H], and [L]). Anterior is oriented toward the top. (A–D) Cells at somite boundaries in wild-type embryos. The arrowheads point to the intersomitic boundary. (E–H) Cells in the presomitic mesoderm (PSM) of wild-type embryos. The arrows point to epithelial adaxial cells in which centrosomes are apically localized (H), as also seen in epithelial cells at somite boundaries (D). Centrosomes are randomly positioned in other PSM cells. (I–L) Cells in the somitic mesoderm of *fss*$^{-/-}$ embryos. n, notochord; ac, adaxial cells. The scale bars represent 10 μm.

[6, 20]. We show that in *fss*$^{-/-}$ embryos, disruption of the Eph/Ephrin signaling interface is accompanied by a failure of paraxial mesoderm cells to undergo the mesenchymal-to-epithelial transition required for somite formation. Using a genetic mosaic approach, we demonstrate that restoration of unidirectional or bidirectional Eph/Ephrin signaling in the paraxial mesoderm of *fss*$^{-/-}$ embryos is sufficient to rescue and maintain morphologically distinct boundaries. Furthermore, many aspects of epithelialization are also rescued in cells at the boundaries induced in *fss*$^{-/-}$ embryos. For instance, activation of EphA4 signaling results in the cell-autonomous acquisition of columnar epithelial morphology and apical relocalization of β-catenin. Conversely, apical localization of the centrosome and basally directed relocalization of the nucleus is a cell-nonautonomous consequence of EphA4 activation. Nuclear relocalization is dependent on Fss but may be independent of Ephrin-reverse signaling; these observations suggest the involvement of a parallel pathway activated by EphA4 signaling. Altogether, these results reveal a pivotal role for Eph receptors and Ephrins as effectors of somite morphogenesis.

Results

Cells of the Paraxial Mesoderm Fail to Undergo Mesenchymal-to-Epithelial Transition in *fss* Mutant Embryos

During somite formation, PSM cells positioned at either side of the prospective intersomitic boundary align to form palisade-like structures along the intersomitic furrow (Figure 1A). Unlike in chick [21], there is virtually no cell movement during this process in fish [22], (see Movie 1 in the Supplemental Data available with this article online). However, the boundary cells do undergo changes associated with transformation from a mesenchymal to an epithelial morphology. Epithelial morphology is revealed by the acquisition of a columnar shape (Figure 1A), accumulation of molecules associated with adhesion complexes, such as β-catenin, at the apical pole of the cells (Figure 1B), basally directed relocalization of cell nuclei toward the somite boundary (Figure 1C), and apical relocalization of centrosomes (Figure 1D). These changes are initiated contemporaneously with, and not prior to, intersomitic boundary formation (see Figure S1 in the Supplemental Data). Unlike cells at the boundaries, those within the core of the somite remain mesenchymal like cells of the PSM.

In *fss* mutants, cells in the maturing somitic mesoderm undergo some reorganization but somite boundaries do not form. Cells fail to epithelialize at segment borders and remain mesenchymal (Figure 1I), β-catenin appears localized homogenously throughout the cell membrane (Figure 1J), nuclei remain in the center of the cells (Figure 1K), and centrosomes are distributed randomly within the cytoplasm (Figure 1L). The morphology of the cells in the somitic mesoderm of *fss* mutants resembles the mesenchymal morphology of cells in the core of the PSM of wild-type embryos (Figures 1E–1H). The PSM of wild-type zebrafish embryos also contains cells with epithelial morphology at sites where the paraxial mesoderm borders with the notochord, neural and surface ectoderm, and lateral plate (Figure S1). In *fss*$^{-/-}$ embryos, equivalent cells also display epithelial morphol-

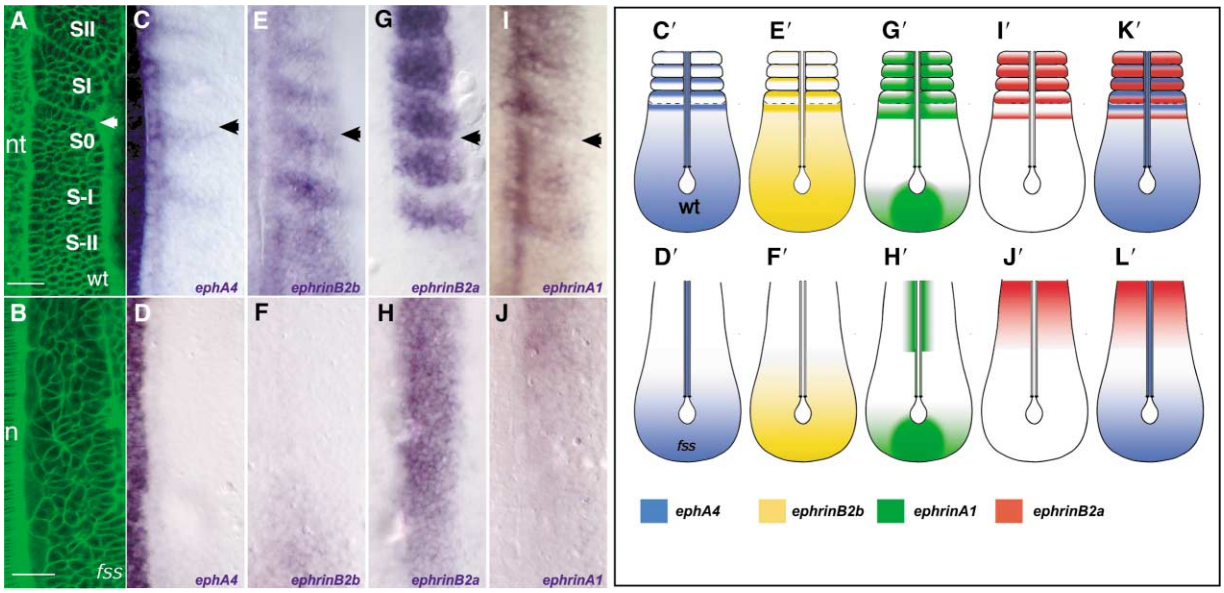

Figure 2. Expression of Eph Family Members in the Paraxial Mesoderm of Wild-Type and *fss*$^{-/-}$ Embryos

Dorsal views of the paraxial mesoderm of 8-somite-stage wild-type and 10-somite-stage *fss*$^{-/-}$ embryos and schematics with anterior oriented toward the top. The arrowheads indicate the position of the most recently formed intersomitic boundary.

(A and B) Living wild-type and *fss*$^{-/-}$ embryos labeled with Bodipy ceramide. In the wild-type embryo, the positions of the last two somites formed (SII, SI), the forming somite (S0), and the two presumptive somites in the PSM (S-I, S-II) are indicated. The arrowhead points to the intersomitic boundary.

(C–J) Expression of (C and D) *ephA4*, (E and F) *ephrin-B2b*, (G and H) *ephrin-B2a*, and (I and J) *ephrin-A1* in wild-type (top row) and *fss* mutant (bottom row) embryos in the region of the paraxial mesoderm shown in (A) and (B).

(C′–J′, K, and L) Schematics summarizing expression of Eph family members in the paraxial mesoderm of wild-type and *fss*$^{-/-}$ embryos.

nt, neural tube; n, notochord. The scale bars represent 30 μm.

ogy (data not shown and [20]), indicating that the intracellular machinery required for cellular epithelialization is functional in *fss*$^{-/-}$ cells.

Eph/Ephrin Signaling Is Disrupted in the Paraxial Mesoderm of *fss*$^{-/-}$ Embryos

In the rostral PSM of wild-type embryos, somite boundary formation is preceded by the segmental expression of EphA4, two Ephrins (Ephrin-B2a and Ephrin-A1) that bind this receptor [18], and Ephrin-B2b, which is also likely to bind EphA4 [10]. *ephA4* and *ephrin-B2b* are expressed in two or three stripes in the PSM [18] such that by the stage that cells are in somite 0 (the somite being formed), expression is restricted to one or two rows of cells in the most anterior region of the segment adjacent to the forming somite boundary (Figures 2C and 2E). *ephrin-B2a* and *ephrin-A1* show graded expression within presumptive somites, and the highest expression is in posterior cells adjacent to the forming boundary (Figures 2G and 2I). Each new intersomitic furrow, therefore, forms at the interface between posterior cells in somite 0, expressing high levels of *ephrin-B2a* and *ephrin-A1*, and anterior cells in somite-I, expressing high levels of *ephA4* and *ephrin-B2b* (Figure 2K).

Segmental expression of these Eph family members is abolished in *fss* mutants. *ephA4* and *ephrin-B2b* expression in cells with anterior identity is absent in the paraxial mesoderm (Figures 2D and 2F), whereas expression of *ephrin-B2a* is detected throughout the somite-II/somite II region (Figure 2H). Segmental expression of *ephrin-A1* is also lost in *fss*$^{-/-}$ embryos, and, in the somite-II/somite II region, transcripts are only detected in the medial region of the paraxial mesoderm (Figure 2J). Analysis of the expression patterns of these Eph family members demonstrates the absence of a ligand-receptor interface in the PSM of *fss* mutants; this lack of interface suggests that Eph/Ephrin signaling is disrupted in the region where somite boundaries should be forming (Figure 2L).

Restoration of the Eph/Ephrin Signaling Interface Rescues the Formation of Morphologically Distinct Boundaries in the Paraxial Mesoderm of *fss*$^{-/-}$ Embryos

To test the hypothesis that loss of *ephA4* expression in the rostral PSM contributes to the failure to form somites in *fss*$^{-/-}$ embryos, we designed a series of experiments to restore the Eph/Ephrin interface in the paraxial mesoderm of *fss*$^{-/-}$ mutants. Wild-type donor cells expressing an EphA4-eGFP fusion protein [6] were transplanted to the prospective paraxial mesoderm of *fss*$^{-/-}$ host embryos. When clusters of wild-type cells expressing exogenous EphA4 were present in the paraxial mesoderm of *fss*$^{-/-}$ embryos, ectopic morphologically distinct boundaries were visible at the interface between EphA4-expressing donor cells and Ephrin-expressing

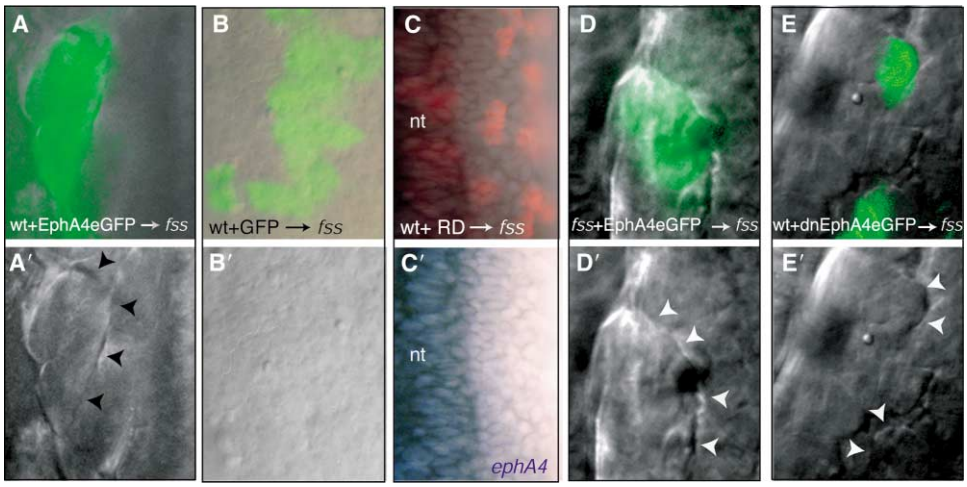

Figure 3. Eph/Ephrin Signaling Restores Morphologically Distinct Boundaries in *fss*$^{-/-}$ Embryos

(A–E and A′–E′) (A–E) DIC and fluorescence overlays and (A′–E′) DIC images of the paraxial mesoderm of *fss*$^{-/-}$ hosts into which wild-type (wt) or *fss*$^{-/-}$ cells expressing various GFP-tagged reagents (green labeling in [A], [B], [D], and [E]) or containing rhodamine dextran (RD, red labeling in [C]) have been transplanted. Reagents are indicated at the bottom of the panels. (C′) *ephA4* expression (blue) is absent from the transplanted cells. The arrowheads point to morphologically distinct boundaries formed at the interface between donor and host cells. nt, neural tube.

fss$^{-/-}$ host cells in 84% (n = 57) of the cases (Figure 3A, Table 1). As irregular and weak boundaries do occasionally form in the paraxial mesoderm of unmanipulated *fss*$^{-/-}$ embryos, we assayed the frequency with which clones of cells might coincidentally align with these boundaries. When *fss*$^{-/-}$ cells were transplanted to the prospective paraxial mesoderm of *fss*$^{-/-}$ host embryos, we found only 14% of the clones aligned with an endogenous boundary (Table 1).

In order to address the possibility that factors other than exogenous EphA4 mediate the rescue of boundaries when wild-type cells are transplanted to *fss*$^{-/-}$ hosts, wild-type donor cells expressing only GFP were transplanted into *fss*$^{-/-}$ hosts. Wild-type cells did not restore boundary formation, and clones coincided with the rare endogenous boundaries in the somitic mesoderm of *fss*$^{-/-}$ hosts at a frequency no greater than we had observed for control transplants of *fss*$^{-/-}$ cells (Figure 3B, Table 1). This was surprising since we expected that cell-autonomous activity of wild-type Fss might promote the endogenous expression of anterior markers including *ephA4* in wild-type cells transplanted to *fss*$^{-/-}$ hosts. However, although wild-type cells do cell-autonomously express various anterior segmental markers when transplanted into *fss*$^{-/-}$ hosts (see Figure S2 in the Supplemental Data), there is no detectable *ephA4* expression (Figure 3C). Together, these results indicate that wild-type cells are neither able to rescue an Eph/Ephrin signaling interface nor formation of morphologically distinct boundaries when transplanted into *fss*$^{-/-}$ hosts.

Fss regulates the expression of many genes in addition to *ephA4*, and so we tested whether exogenous EphA4 requires the activity of other Fss-dependent proteins to rescue the formation of morphologically distinct boundaries. When clusters of EphA4-expressing *fss*$^{-/-}$ cells were present in the somitic mesoderm of *fss*$^{-/-}$ hosts, ectopic, morphologically distinct boundaries formed at the interface between donor and host cell populations in 87% (n = 23) of the cases (Figure 3D, Table 1). These results demonstrate that boundaries can be induced in *fss*$^{-/-}$ embryos solely by the restoration of an Eph/Ephrin signaling interface.

Our favored interpretation of these results is that Eph/Ephrin signaling functions as one of the final steps in boundary formation. An alternative possibility is that exogenous EphA4 signaling restores expression of other genes that function in the anterior region of the forming somite and that it is these other factors that mediate boundary formation. However, this is unlikely, as wild-type cells with anterior character (but lacking *ephA4* expression) do not induce boundaries (Figure 3B), expression of several anterior markers appears to be unaffected by EphA4 activity, and exogenous EphA4 can still promote boundary formation when Notch signaling is disrupted (Figure S2). These observations suggest that Eph/Ephrin signaling directly mediates boundary formation downstream of the acquisition of "anterior" identity and does not induce boundaries by changing the fate of presomitic cells.

Table 1. Rescue of Boundary Formation in Mosaic Experiments

Experiment	Boundary Formation	n
fss → fss	14%	7
wt → fss	4%	24
wt + EphA4 → fss	84%	57
fss + EphA4 → fss	87%	23
wt + dnEphA4 → fss	93%	29
wt + EphA4 → fss + dnEphrin-B2a	86%	22

n is the total number of fss host embryos analyzed. Data are pooled from several independent experiments. wt, wild-type; fss, fused somites.

Transplanting EphA4-expressing cells into *fss*$^{-/-}$ embryos is likely to activate signaling downstream of both receptor-expressing donor cells and Ephrin-expressing host cells. To test whether signaling downstream of Ephrins is sufficient to restore the formation of morphologically distinct boundaries, we activated Ephrins with a tagged version of a dominant-negative EphA4 receptor that lacks the intracellular tyrosine kinase domain (see the Experimental Procedures). Truncated membrane bound Eph receptors are able to bind and promote clustering and activation of their counterpart ligands in adjacent cells [23, 24]; however, the lack of a tyrosine kinase domain renders the receptor incapable of transducing an intracellular signal. When wild-type cells expressing truncated EphA4 receptor were transplanted into the somitic mesoderm of *fss*$^{-/-}$ embryos, ectopic boundaries were rescued at the interface between donor and host cell populations in 93% (n = 23) of the cases (Figure 3E, Table 1). This result suggests that activation of Ephrin reverse signaling in cells on one side of the nascent boundary is sufficient to generate morphologically distinct boundaries in the paraxial mesoderm of *fss*$^{-/-}$ embryos.

Boundaries Established by the Restoration of EphA4/Ephrin Signaling in *fss* Embryos Mature Normally during Muscle Differentiation

A major consequence of somite boundary formation is the alignment of muscle fiber attachment sites at the intersomitic boundary (Figure 4A). To assess if morphologically distinct boundaries that form as a consequence of Eph/Ephrin signaling in *fss*$^{-/-}$ mutants resemble wild-type intersomitic furrows, we assayed muscle fiber organization in *fss*$^{-/-}$ embryos with and without transplants of EphA4-expressing cells. In *fss*$^{-/-}$ embryos, there is no precise alignment of muscle fibers. Even when rare, irregular, and aberrantly shaped endogenous boundaries are present, muscle fibers still frequently cross the boundaries (Figure 4B).

The morphologically distinct boundaries that form in *fss*$^{-/-}$ embryos following unidirectional or bidirectional Eph/Ephrin signaling from wild-type or *fss*$^{-/-}$ donor cells are capable of organizing muscle fibers. Both host and donor muscle fiber attachment sites are aligned at the induced boundaries, and muscle fibers do not extend across the boundary into adjacent cell populations. Muscle fibers in EphA4-expressing clusters of wild-type cells (Figure 4C) appear more compact and better aligned than those in EphA4-expressing *fss*$^{-/-}$ cell clusters (Figure 4D) or in truncated EphA4-expressing wild-type cell clusters (Figure 4E). This suggests that both signaling downstream of the receptor and other Fss-dependent factors contribute to the proper morphogenesis and differentiation of the clones (see below and the Discussion).

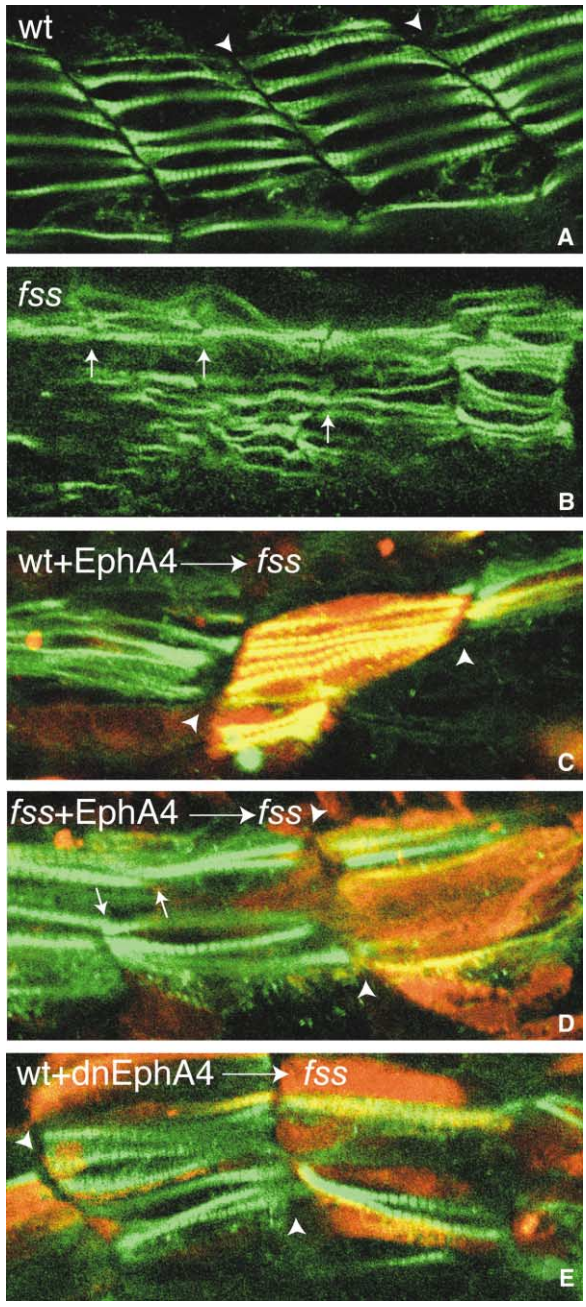

Figure 4. Boundaries Restored by Eph/Ephrin Signaling Are Maintained during Muscle Differentiation

(A–E) Lateral views of (A) wild-type and (B–E) *fss*$^{-/-}$ somitic muscles immunostained for myosin (green). Anterior is oriented toward the left. (C), (D), and (E) show transplanted (C and E) wild-type or (D) *fss*$^{-/-}$ cells (red) expressing (C and D) full-length or (E) truncated, dominant-negative EphA4. The arrowheads point at morphologically distinct furrows. The arrows point at *fss*$^{-/-}$ muscle fibers that span adjacent segments.

Apical Distribution of β-Catenin and Acquisition of Columnar Morphology Are Downstream Consequences of EphA4 Signaling

In addition to the formation of a morphologically distinct boundary, somite morphogenesis leads to epithelialization of cells at the boundary, a process that fails to occur in *fss*$^{-/-}$ mutants (Figure 1). To investigate whether Eph/Ephrin signaling is involved in mediating this mesenchymal-to-epithelial transition, we analyzed whether cells along boundaries induced by Eph/Ephrin signaling in *fss*$^{-/-}$ embryos became epithelialized.

In 70% (n = 20) of the cases in which morphologically

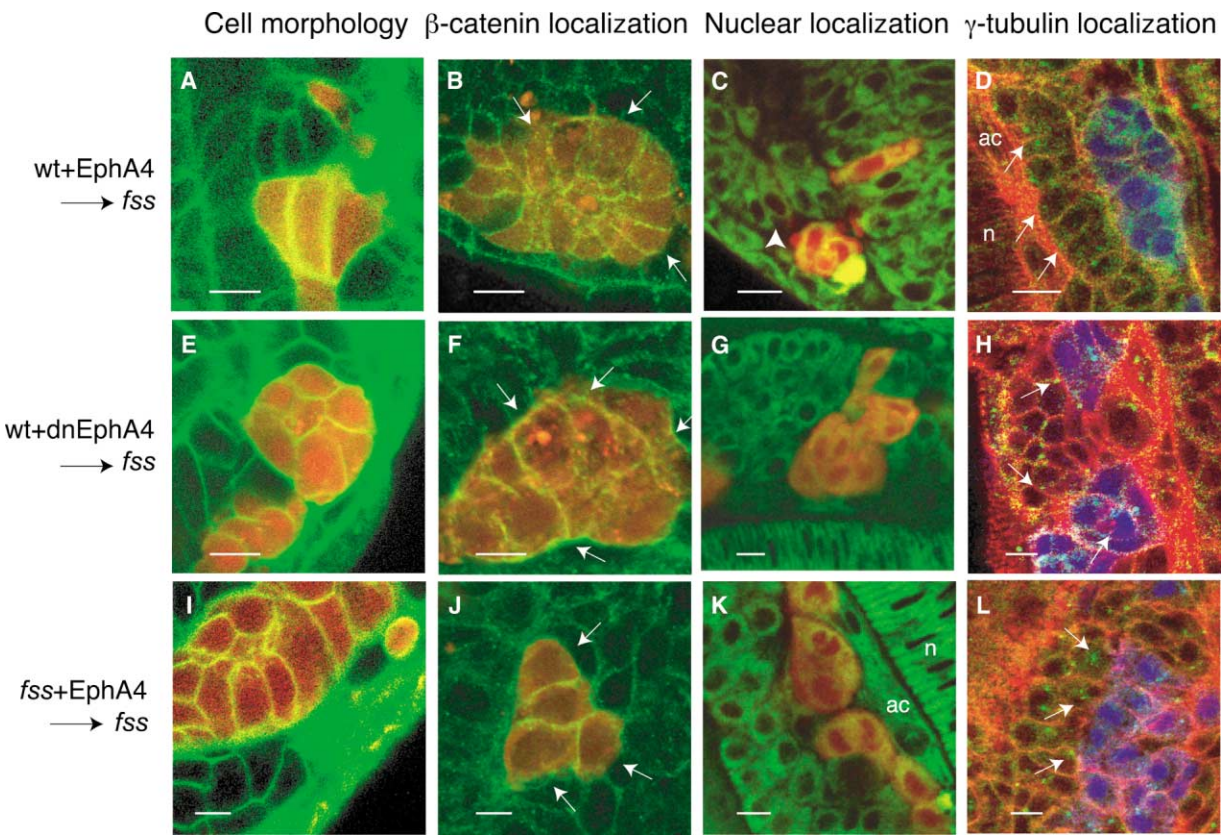

Figure 5. Eph/Ephrin Signaling Rescues Epithelialization of Cells at Morphologically Distinct Boundaries

(A–L) (A, E, and I) Confocal images showing Bodipy ceramide-labeled somitic mesoderm of *fss*$^{-/-}$ host embryos (green) containing rhodamine dextran-labeled donor cells (red). (B, F, and J) Confocal images of β-catenin immunolocalization (green) in transplanted rhodamine dextran-labeled cells (red) in *fss*$^{-/-}$ host embryos. The arrows point to the basal surfaces of the transplanted cells at the interface at which morphologically distinct boundaries form (visible with DIC optics, not shown). In (B) and (J), β-catenin is reduced on the basal surfaces of these cells. (C, G, and K) Confocal images showing Bodipy 505-515-labeled somitic mesoderm of *fss*$^{-/-}$ host embryos (green) containing rhodamine dextran-labeled donor cells (red). The white arrowhead points to nuclei localized at the basal pole of host *fss*$^{-/-}$ cells, adjacent to the boundaries created between donor and host cells. (D, H, and L) Confocal images showing phalloidin-labeled somitic mesoderm of *fss*$^{-/-}$ host embryos (red) containing CFP-labeled donor cells (blue) and immunostained for γ-tubulin (green). The white arrows point to centrosomes. These are localized at the apical pole of host *fss*$^{-/-}$ cells adjacent to the boundary created between donor and host cells in (D) but are randomly positioned in (H) and (L). Donor cells are wild-type cells expressing (A–D) full-length or (E–H) truncated, dominant-negative EphA4 or *fss*$^{-/-}$ cells expressing (I–L) full-length EphA4. n, notochord; ac, adaxial cells. The scale bars represent 10 μm.

distinct boundaries were established at the interface between *ephA4*-expressing, wild-type donor cells and *ephrin*-expressing *fss*$^{-/-}$ host cells, the donor cells at the boundary acquired a columnar (see the Experimental Procedures) morphology (Figure 5A, Table 2). In 80% (n = 15) of the cases, donor cells were judged to show increased levels of β-catenin at the apical pole of the cell and reduced levels basally (Figure 5B, Table 2). To investigate if this epithelialization is dependent upon EphA4 interacting with other Fss-dependent factors, we analyzed whether epithelialization of *fss*$^{-/-}$ cells occurs following activation of EphA4. In 70% (n = 19) of the cases, EphA4-expressing *fss*$^{-/-}$ cells relocalized β-catenin toward the apical pole when transplanted next to Ephrin-expressing *fss*$^{-/-}$ host cells (Figure 5J, Table 2). In seven of nine cases, donor *fss*$^{-/-}$ cells acquired a columnar morphology (Figure 5I, Table 2).

Although these results suggest that EphA4 signaling

Table 2. Rescue of Epithelialization in Mosaic Experiments

	Donor Cells		Host Cells	
Experiment	β-Catenin Relocalization	Columnar Morphology	Nuclear Relocalization	Columnar Morphology + Centrosome Relocalization
Wt + EphA4 → fss	80% (n = 15)	70% (n = 20)	52% (n = 49)	50% (n = 14)
Wt + dnEphA4 → fss	7% (n = 14)	8% (n = 12)	7% (n = 29)	0% (n = 6)
Fss + EphA4 → fss	70% (n = 19)	77% (n = 9)	13% (n = 23)	11% (n = 9)

n is the total number of fss host embryos analyzed. Data are pooled from several independent experiments. wt, wild-type; fss, fused somites.

mediates epithelialization, an alternative possibility is that Eph signaling establishes a boundary and that as a consequence of boundary formation, other EphA4-independent events lead to epithelialization. To address if this may be the case, we transplanted wild-type cells expressing truncated EphA4, which we know can induce boundaries but cannot transduce intracellular signaling downstream of EphA4. Although truncated EphA4 induces boundary formation, cells do not adopt a columnar morphology and β-catenin remains throughout the membrane, including at the basal pole adjacent to the boundary (Figures 5E and 5F, Table 2). These results indicate that cell shape changes and β-catenin relocalization are dependent on cell-autonomous activation of EphA4 signaling and are not secondary consequences of boundary formation.

Epithelialization of Host *fss*$^{-/-}$ Cells Is a Cell-Nonautonomous Effect of EphA4 Signaling Dependent on Fss

When wild-type cells expressing exogenous EphA4 were present in the somitic mesoderm of *fss*$^{-/-}$ hosts, 84% (n = 57) of the cell groups induced boundary formation. In 52% of these cell groups (44% of the total number of clones), the nuclei of Ephrin-expressing *fss*$^{-/-}$ host cells became localized at the basal pole, toward the boundary (Figure 5C, Table 2). Basal nuclear relocalization in *fss*$^{-/-}$ host cells was accompanied by the acquisition of a columnar morphology and apical relocalization of the centrosome (Figures 5A and 5D, Table 2). Epithelialization of host *fss* cells is, therefore, a cell-nonautonomous effect of EphA4 activity. Despite acquiring these features of epithelialization, Ephrin-expressing host cells showed no evidence of apical β-catenin relocalization (not shown). Similarly, relocalization of the nucleus and the centrosome was not observed in donor EphA4-expressing cells (not shown). Together, these results indicate that while epithelial morphology is rescued on both sides of the Eph/Ephrin-induced boundaries, more subtle aspects of cell polarity may remain disrupted.

Given that Ephrins can signal intracellularly, a cell-nonautonomous effect of the receptor suggests a role for Ephrin reverse signaling in epithelialization. We therefore performed several sets of experiments to elucidate the requirement for Ephrin signaling in cell elongation and nuclear relocalization. First, we examined cell shape and cell polarity in *fss*$^{-/-}$ host cells at boundaries induced by transplantation of truncated EphA4-expressing wild-type donor cells. Truncated EphA4 is able to activate Ephrin reverse signaling in adjacent cells but is unable to signal intracellularly. Although 93% (n = 29) of the clones formed morphologically distinct boundaries, no obvious rescue of cell elongation, basal nuclear relocalization, or apical relocalization of the centrosome was observed in the adjacent Ephrin-expressing *fss*$^{-/-}$ host cells (Figures 5E, 5G, and 5H, Table 2). This result indicates that the reverse signaling induced by truncated EphA4 is not sufficient to promote cell epithelialization. As full-length, but not truncated, EphA4 can nonautonomously restore epithelial morphology in adjacent cells, this result also implies that intracellular signaling downstream of EphA4 is important in this event.

Next, we attempted to more directly address whether reverse signaling downstream of Ephrins is required for epithelialization. To do this, we overexpressed an intracellularly truncated dominant-negative form of Ephrin-B2a throughout the somitic mesoderm of *fss*$^{-/-}$ mutant hosts. Truncated Ephrin-B2a is able to bind and activate EphA4 in adjacent cells but is unable to signal intracellularly and is likely to suppress endogenous Ephrin-B reverse signaling [17, 18] (and see the Experimental Procedures). When clones of wild-type donor cells expressing EphA4 were transplanted into truncated Ephrin-B2a-expressing *fss*$^{-/-}$ hosts, the acquisition of columnar morphology and nuclear relocalization still occurred in host cells in 57% of the cases in which morphologically distinct boundaries formed (n = 22; see Figure S3A in the Supplemental Data). This frequency is not significantly different from that seen in experiments in which reverse Ephrin signaling was unperturbed. Together, these results demonstrate that elongation and polarization of *fss*$^{-/-}$ host cells are cell-nonautonomous effects downstream of EphA4 signaling for which Ephrin reverse signaling may not be essential.

These observations suggest the presence of a parallel pathway that becomes activated by EphA4 signaling in the receptor-bearing cells and that signals back to adjacent *fss*$^{-/-}$ cells to promote cell elongation and nuclear migration. To investigate whether this parallel pathway is fully functional in *fss*$^{-/-}$ mutants, we transplanted EphA4-expressing *fss*$^{-/-}$ cells into *fss*$^{-/-}$ hosts and assessed epithelialization in the host cells. Whereas *fss*$^{-/-}$ host cells undergo elongation and relocalization of cellular organelles in response to EphA4-expressing wild-type donor cells, no significant epithelialization was seen when the donor cells lacked Fss activity (Figures 5I–5L, Table 2). These results indicate that the factor/s that cooperates with EphA4 in the receptor-bearing cells is not present or is functionally compromised in *fss*$^{-/-}$ cells.

Discussion

During somite morphogenesis, a furrow of de-adhesion creates a boundary between the populations of paraxial mesodermal cells that will form adjacent somites. Cells on both sides of the forming somite boundary undergo a mesenchymal-to-epithelial transition that involves changes in cell shape, cell adhesive interactions, and subcellular polarization of organelles and proteins. Here, we have presented several lines of evidence that Eph/Ephrin signaling has key roles in boundary formation and somite morphogenesis. First, in embryos that lack somites, restoration of Eph/Ephrin signaling interfaces rescues the formation and subsequent maturation of morphologically distinct boundaries. Second, activation of EphA4 signaling both cell-autonomously and -nonautonomously rescues various aspects of somite boundary cell epithelialization.

An Eph/Ephrin Interface Is Required for Morphological Segmentation of the Paraxial Mesoderm

We have previously demonstrated that disrupting Eph/Ephrin signaling in the paraxial mesoderm of wild-type zebrafish embryos disturbs the formation of somites.

These findings implicate Eph/Ephrin signaling in somitogenesis [18]. In this study, we have elucidated the events for which Eph/Ephrin signaling is required through analysis of the *fss* mutant, which lacks organized somites.

Although several signaling pathways could potentially be disrupted in *fss* mutants, our data show that loss of somite boundaries is most likely due to the absence of Eph/Ephrin signaling interfaces. The absence of *ephA4* expression in the PSM of *fss* mutants means a loss of the interfaces between EphA4-expressing and EphrinB2a/EphrinA1-expressing cells that normally occur between anterior and posterior cells in adjacent segments. Restoration of Eph/Ephrin signaling in the paraxial mesoderm by the apposition of either wild-type or $fss^{-/-}$ cells expressing EphA4 with Ephrin-expressing $fss^{-/-}$ host cells results in the rescue of morphologically distinct boundaries. In the case of $fss^{-/-}$ cells expressing EphA4, this rescue occurs without restoring the expression of other markers of anterior segmental identity in the donor cells. Conversely, apposition of wild-type cells, which lack detectable *ephA4* expression, with $fss^{-/-}$ cells does not result in boundary formation, despite the fact that wild-type cells do express anterior segmental markers other than *ephA4*. These results demonstrate that the presence of EphA4 is sufficient to restore boundaries to the paraxial mesoderm of $fss^{-/-}$ embryos. Furthermore, they imply that Eph/Ephrin signaling mediates the final step of somite boundary formation, downstream of the acquisition of anterior or posterior segmental character.

An intracellularly truncated form of EphA4 can also restore boundaries in $fss^{-/-}$ embryos. This suggests that signaling downstream of the receptor is not essential and that Ephrin reverse signaling is sufficient to induce the formation of a physical furrow between adjacent cell populations. In contrast, bidirectional Eph/Ephrin signaling is required to restrict cell intermingling and boundary formation in blastomere intermixing assays [15]. However, in these assays, cells normally intermingle extensively, whereas in the rostral PSM, there is hardly any cell movement and cell mixing does not occur. Therefore, it may be that in situations in which cell movements are limited, unidirectional Eph/Ephrin signaling is sufficient to induce boundary furrow formation. Similarly, unidirectional Eph/Ephrin signaling is sufficient to restrict cell movement within hindbrain rhombomeres [16].

In addition to initiating boundary formation, restoration of Eph/Ephrin signaling leads to the appropriate alignment of muscle fiber attachment sites at these boundaries, even in situations in which the initial epithelialization of boundary cells fails to occur. Therefore, epithelialization is not an absolute prerequisite for the maturation of somite boundaries. However, it may be important for the correct organization and compaction of muscle fibers since these features were more completely rescued in situations where boundary cells had undergone epithelialization.

EphA4 Signaling Leads to Epithelialization of Boundary Cells

During epithelialization, cells acquire a columnar morphology and form apically positioned cadherin/catenin-containing adherens junctions [25]. Both wild-type and $fss^{-/-}$ cells expressing exogenous EphA4 acquire a columnar morphology and show apical localization of β-catenin when transplanted into the paraxial mesoderm of $fss^{-/-}$ mutants. These results suggest that these aspects of epithelialization are a consequence of activation of EphA4 signaling. In support of this interpretation, expression of intracellularly truncated EphA4 does not lead to the acquisition of a columnar morphology or β-catenin relocalization, despite the fact that it is able to induce the formation of morphologically distinct boundaries. Therefore, epithelialization is not simply a consequence of the formation of boundaries between paraxial mesodermal cells. A similar conclusion has been reached from analysis of mice lacking function of the transcription factor Paraxis [26]; in these mice, the paraxial mesoderm exhibits intersomitic furrows, but cells at the boundaries fail to become epithelial. Together, these results suggest that boundary furrow formation is an early event in the partitioning of the paraxial mesoderm and that subsequent morphological changes within the cells adjacent to the boundary require additional signals.

Further features of the epithelialization of somite cells are the basally directed relocalization of the nucleus and the apical relocalization of the centrosome. Perhaps surprisingly, relocalization of the cellular organelles was uncoupled from some other aspects of epithelialization and occurred in host $fss^{-/-}$ mutant cells only when they were confronted with EphA4-expressing wild-type cells. Therefore, these aspects of epithelialization are a cell-nonautonomous consequence of EphA4 signaling. Cell-nonautonomous effects of Eph receptors are generally thought to be independent of the intracellular domain of the receptor and are usually attributed to reverse signaling downstream of Ephrins expressed in adjacent cells (see [27] for a review). However, nuclear relocalization in $fss^{-/-}$ host cells requires the intracellular domain of EphA4 and may be independent of Ephrin reverse signaling. One possible explanation of these observations is that a parallel pathway is activated downstream of EphA4 signaling in the receptor-bearing cells that triggers nuclear relocalization in adjacent cells. Since Eph receptors are known to interact with other transmembrane receptors during synapse formation, craniofacial development, and neural connectivity (reviewed in [9]), it is plausible that cross-talk is also occurring during somite epithelialization. Nevertheless, a role for Ephrin reverse signaling in epithelialization cannot be ruled out since the intracellular domain of the receptor may be required to fully activate Ephrin reverse signaling in adjacent cells.

Several lines of evidence led us to investigate the possibility that the protocadherin Papc may be a component of a pathway cooperating with EphA4 in promoting epithelialization (Figure S3). However, Papc (in the presence of EphA4) is neither sufficient in $fss^{-/-}$ cells nor likely to be required in wild-type cells to promote epithelialization of adjacent $fss^{-/-}$ cells (Figure S3). Alternative candidates for proteins that interact with Eph receptors to promote epithelialization include the integrin family of transmembrane receptors. Although extracellular matrix molecules are the classical ligands for integrins, mem-

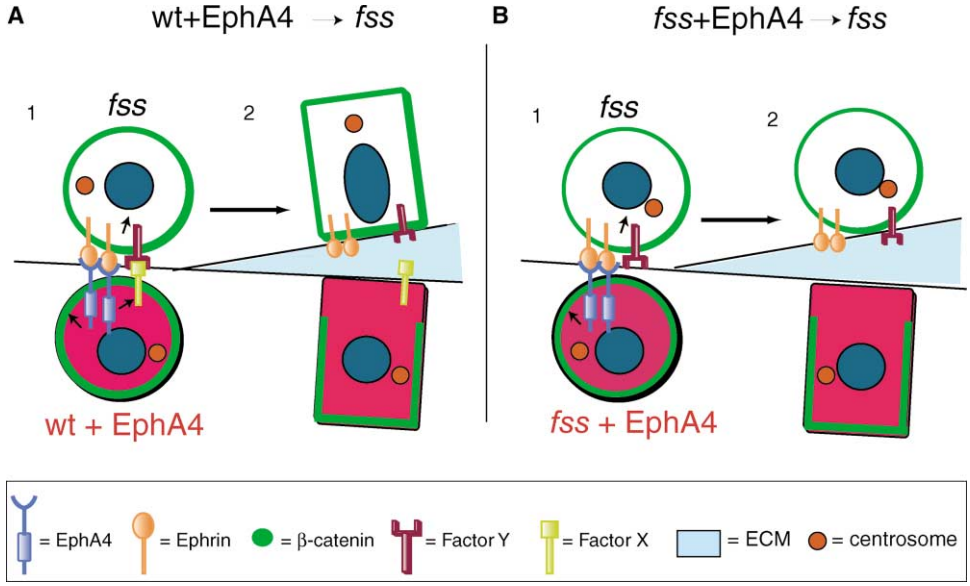

Figure 6. Summary Model of Roles for Eph/Ephrin Signaling during Somite Epithelialization

The red circles or rectangles represent donor wild-type or *fss*$^{-/-}$ cells during (circles) and after (rectangles) epithelialization. The white circles or rectangles represent host *fss*$^{-/-}$ cells. Although Ephrins (orange) are represented as transmembrane molecules, GPI-linked Ephrins may also play a role. The cofactor (yellow) interacting with EphA4 in donor cells is represented as a transmembrane molecule; however, it could be a secreted molecule or other form of protein.

(A1 and B1) Donor cells expressing EphA4 (blue) and host cells expressing interacting Ephrins (orange) are in contact within the paraxial mesoderm. This allows for Eph/Ephrin binding and activation. Both cells present mesenchymal morphology. β-catenin (green) is homogenously distributed throughout the cell membrane, the nucleus is localized in the center of the cell body, and the centrosome (red) is localized randomly within the cytoplasm.

(A2 and B2) Activation of Ephrin reverse signaling leads to localized de-adhesion and boundary formation between both cells. Activation of EphA4 signaling leads to relocalization of β-catenin toward the apical pole of the cell, cell elongation, and boundary formation. (A2) In wild-type cells, EphA4 signaling also leads to the activation of factor X (yellow) in donor cells and its interaction with factor Y (brown) in adjacent host cells. Signaling downstream of factor Y results in the basal relocalization of the nucleus toward the boundary, the apical relocalization of the centrosome, and the acquisition of a columnar morphology. (B2) In *fss*$^{-/-}$ donor cells, where factor X is not present or has compromised function, signaling downstream of factor Y in adjacent *fss*$^{-/-}$ host cells does not occur and they remain mesenchymal, despite boundary formation.

brane bound ligands also exist (see [28] for a review). In mouse embryos lacking α5-integrin, the paraxial mesoderm segments, but epithelial somites fail to form [29], suggesting a role for integrin signaling in somite formation. Furthermore, roles for Eph receptor signaling in the regulation of integrin activity have been suggested in other situations [30–36]. At present, little is understood about the biochemical interactions between Eph receptors/Ephrins and integrins, but it is clearly an area in need of further exploration.

Eph Receptors and Ephrins as Effectors of Somite Morphogenesis

We present a model to explain our results and to predict how Eph/Ephrin signaling regulates boundary formation and epithelialization (Figure 6). In the PSM of *fss*$^{-/-}$ chimaeras, cells expressing exogenous EphA4 are in close proximity to cells expressing endogenous Ephrin ligands, with the consequence that receptors and ligands bind, cluster, and become activated. Events downstream of the Eph receptor and Ephrin activation lead to local de-adhesion and boundary formation. Coincident with furrow formation, EphA4 protein is removed from the cell surface facing the boundary (see Figure S4 in the Supplemental Data); this removal of protein presumably terminates Eph/Ephrin signaling. Signaling downstream of EphA4 subsequently leads to the acquisition of a columnar morphology and apical accumulation of β-catenin in a cell-autonomous manner. Activation of EphA4 signaling also leads to the activation of a parallel pathway that promotes the acquisition of columnar morphology and polarized relocalization of the nucleus and the centrosome in adjacent cells (Figure 6A). In *fss*$^{-/-}$ cells, components of the parallel pathway involved in stimulating adjacent host cells lack function, and therefore, despite expressing EphA4, *fss*$^{-/-}$ cells are unable to rescue epithelialization in the adjacent cell population (Figure 6B).

EphA4 promotes different aspects of epithelialization in two different cell populations: β-catenin redistribution occurs in receptor-bearing cells, whereas relocalization of the nucleus and the centrosome occur in ligand-expressing cells. However, in wild-type embryos, cells on both sides of the somite boundary undergo all aspects of epithelialization. One possible explanation of why cells expressing exogenous EphA4 do not undergo relocalization of the subcellular organelles may be that overexpression of the receptor leads to a gain-of-function phenotype and not to the phenotype that is a result

of normal activation of EphA4. Indeed, ectopic EphA4 catalytic activity in *Xenopus* embryos leads to the loss of cell polarity in early blastula cells [37]. In our experiments, however, activation of EphA4 in wild-type or *fss*$^{-/-}$ cells does lead to the acquisition of columnar morphology and the polarized distribution of β-catenin, aspects of epithelialization characteristic of cells at wild-type somite boundaries. Therefore, our data are consistent with the possibility that exogenous EphA4 recapitulates the normal activity of the receptor and that nuclear relocalization is a cell-nonautonomous effect of EphA4 signaling.

To extrapolate our results to a wild-type situation where all aspects of epithelialization occur in cells on both sides of the intersomitic boundary, we need to postulate that both cell populations express receptor and ligand. Although a receptor expressed in the posterior domain of the forming somite has not yet been identified in zebrafish, several Eph proteins that could fulfill this role are known in other species [38–41]. Based on these observations, we think it highly likely that the posterior region of the forming somite expresses an Eph receptor yet to be identified in fish. This receptor could then mediate β-catenin relocalization in posterior cells and relocalization of cellular organelles in anterior cells on the other side of the forming somite boundary.

Conclusions

The results presented in this paper indicate that Eph/Ephrin signaling in the rostral PSM is an important component of the molecular machinery that drives somite morphogenesis. Restoration of EphA4/Ephrin signaling in the paraxial mesoderm of *fss*$^{-/-}$ mutants is sufficient to rescue the formation of morphologically distinct boundaries. Moreover, activation of EphA4 signaling results in the mesenchymal-to-epithelial transition in the morphology of boundary cells and thereby recapitulates most aspects of somite morphogenesis. However, restoration of all aspects of epithelialization is likely to additionally require the activity of pathways acting in parallel to Eph/Ephrin signaling.

Experimental Procedures

Maintenance of Fish

Breeding zebrafish (*Danio rerio*) were maintained at 28°C on a 14 hr light/10 hr dark cycle. Wild-type and mutant embryos were collected by natural spawning and were staged according to Kimmel et al. [42]. Mutant embryos were generated from fish carrying the *te314a* or *ti1* alleles of *fused somites* [20].

In Situ Hybridization and Immunocytochemistry

Single whole-mount in situ hybridization was performed by following the protocol of Thisse et al. [43]. For muscle labeling, antibody staining was performed essentially as previously described [44], by using pan-myosin antibody 1025 (a gift from Simon Hughes) at 1:2.5 dilution. For β-catenin and γ-tubulin immunostaining, embryos were fixed in 4% PFA for 2 hr at room temperature. For γ-tubulin, embryos were permeabilized by acetone treatment at −20°C for 5 min and were then washed in PBT (0.8% Triton X-100 in PBS). For β-catenin, embryos were permeabilized by methanol treatment at −20°C and were then washed in PDT (PBT + 1% DMSO). Monoclonal anti-β-catenin antibody (BD Transduction Laboratories) was used at 1:500 dilution. Monoclonal anti-γ-tubulin antibody (Sigma) was used at 1:200 dilution. Alexa fluor 488 anti-mouse IgG (Molecular Probes) was used as a secondary antibody at 1:200 dilution. Phalloidin (Molecular Probes) staining was performed after antibody staining. Embryos were incubated for 2 hr at room temperature by using a dilution of 1:40 (in 2% PBT) from the commercial stock.

Cloning, Synthesis, and Injection of mRNA

As previously described [6], the EphA4eGFP fusion construct was made by inserting full-length *Xenopus* EphA4 into the pEGFP-N1 vector (Clontech) upstream of, and in frame with, the GFP coding sequence. The coding sequence for the fusion protein was subsequently cloned into the pCS2 vector for in vitro transcription. The same procedure was used for truncated EphA4eGFP and truncated EphrinB2a. EphA4 was truncated after amino acid 602 by using PCR primers (5′ primer, 5′-TCAGATCTGCCACCATGGCTGGGATTGTA-3′; 3′ primer, 5′-ATCCCGGGATTCATAAGTAAATGGGTC-3′). EphrinB2a was truncated after amino acid 251 by using PCR primers (5′ primer, 5′-TACCGCGGACCATGGGCGACTCT-3′; 3′ primer, 5′-GTGGATCCCGTCGTCGATACTTCAGGAG-3′) [18]. Capped mRNA was synthesized as previously described [18], and 400 pl was injected into embryos at the 1- or 2-cell stage. The concentrations at which the different mRNAs were injected were 300–400 ng/μl for full-length EphA4eGFP and 200 ng/μl for truncated EphA4eGFP and truncated Ephrin-B2eGFP. At these concentrations, truncated EphA4eGFP and truncated Ephrin-B2eGFP disrupted somite boundary formation in wild-type embryos (data not shown).

Mosaic Analysis

Mosaic experiments were performed as previously described [6]. Donor embryos were injected with mRNA encoding GFP fusion proteins or a mixture of RNA encoding GFP or CFP and the mRNA of interest. Donor cells were selected for transplantation by green fluorescence to ensure that they were expressing the injected mRNAs. In some experiments, donors were coinjected with fixable rhodamine dextran (Molecular Probes) to allow visualization of the transplanted cells in host embryos stained with green dyes.

Visualization of Cell Morphology in Living Embryos

Living embryos at stages between 8 and 12 somites were incubated in Bodipy 505-515 or Bodipy ceramide (Molecular Probes) by following the protocol of Cooper et al. [45]. Embryos were mounted in agarose for visualization under confocal microscopy by using an argon laser and the FITC filter set at a 488-nm excitation wavelength. Cells were considered of columnar morphology when the height/width ratio was between 2.3 and 3, compared to an average height/width ratio of 1.3 for mesenchymal cells.

Supplemental Data

Supplemental Data including one movie and four additional figures with discussion are available at http://www.current-biology.com/cgi/content/full/13/18/1571/DC1/.

Acknowledgments

We thank Patrick Blader, in whose laboratory some of this work was done, Stephen Nurrish for his patience and support, Simon Hughes for providing the myosin antibody, Sharon Amacher for providing the Papc constructs, Ajay Chitinis for the XDelSTU construct, and Julie Cooke for comments on the manuscript. We would like to thank members of our group for discussions on this work, Daniel Ciantar for his help with confocal microscopy, Carole Wilson and her colleagues for care of the fish, and many colleagues in the community for provision of various reagents. This work was supported by grants from the Wellcome Trust and Biotechnology and Biological Sciences Research Council to S.W.W. and N.H. S.W.W. is a Wellcome Trust Senior Research Fellow.

Received: March 14, 2003
Revised: July 2, 2003
Accepted: July 25, 2003
Published: September 16, 2003

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